Gene expression in vessel-associated cells upon xylem embolism repair in Vitis vinifera L. petioles.

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Title: Gene expression in vessel-associated cells upon xylem embolism repair in *Vitis vinifera* L. petioles

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Abstract

In this work, the involvement of vessel-associated cells in embolism recovery was investigated by studying leaf petiole hydraulics and expression profiles of aquaporins and genes related to sugar metabolism.

Two different stress treatments were imposed onto grapevines to induce xylem embolism: one involved a pressure collar applied to the stems, while the other consisted of water deprivation (drought). Embolism formation and repair were monitored during stress application and release (recovery). At the same time, stomatal conductance (g_s), leaf water potential (Ψ_{leaf}), and leaf abscisic acid (ABA) concentration were measured. For each treatment, gene transcript levels were assessed on vessel-associated cells (isolated from leaf petioles by laser microdissection technique) and whole petioles.

Both treatments induced severe xylem embolism formation and drops in g_s and Ψ_{leaf} at a lesser degree and with faster recovery in the case of application of the pressure collar. Leaf ABA concentration only increased upon drought and subsequent recovery. Transcripts linked to sugar mobilisation (encoding a β-amylase and a glucose-6-P transporter) were over-expressed upon stress or recovery both in vessel-associated cells and whole petioles. However, two aquaporin genes (*VvPIP2;1* and *VvPIP2;4N*) were activated upon stress or recovery only in vessel-associated cells, suggesting a specific effect on embolism refilling. Furthermore, the latter gene was only activated upon drought and subsequent recovery, suggesting that either severe water stress or ABA are required for its regulation.

Keywords: Aquaporins, Drought, Grapevine, Laser microdissection, Pressure collar, Sugar metabolism.

Abbreviations: ABA (Abscisic acid), IRR (Irrigated), WS (Water stress), RWS (Recovery from water stress), PC (Pressure collar), RPC (Recovery from pressure collar), HCFM (Hydraulic Conductance Flow Meter), LMD (Laser MicroDissection), RT-qPCR (Quantitative Real-Time PCR), VACs (Vessel Associated Cells).

Introduction

Vascular plants have evolved a long-distance transport system for water and minerals through non-living xylem vessels. Long distance transport is driven by tension, as postulated by the Cohesion-Tension theory (Tyree 2003) and further confirmed by direct measurements of negative pressures in xylem (Angeles et al. 2004). Under high tension (e.g., upon drought stress), water is metastable and, when gas-filled, xylem vessels may become disrupted by breakage of water column continuity (cavitation), thus causing embolism that drastically reduces the hydraulic conductance of xylem (in grapevine, Schultz and Matthews 1988; Lovisolo and Schubert 1998; Tramontini et al. 2013). However, many aspects concerning the biophysics of embolism formation in plants remain unclear (Clearwater and Goldstein 2005). Several studies suggested that xylem cavitation is caused by environmental stress, such as drought (e.g., Tyree et al. 1994; Davis et al. 2002) and freezing temperatures (e.g., Just and Sauter 1991; Nardini et al. 2000; Sakr et al. 2003). Nevertheless, cavitation is also a daily cyclical phenomenon occurring even in well-watered plants (Holbrook et al. 2001; Lovisolo et al. 2008; Zufferey et al. 2011).

Xylem embolisms can be refilled (recover) when xylem tension drops to values close to zero. However, embolism recovery takes place also upon tension, and plant metabolism plays an essential role in these conditions, as demonstrated by the effect of metabolic inhibitors (Salleo et al. 1996, 2004; Lovisolo and Schubert 2006). Furthermore, modifications of transcriptional profiles observed upon embolism recovery (Brodersen et al. 2010, 2013; Secchi and Zwieniecki 2010, 2011; Perrone et al. 2012b) suggest that plants can mount specific responses to xylem embolism. Different models have been proposed to explain how plants induce an embolism-refilling process, most of which include key roles for living parenchyma cells surrounding xylem vessels (Vessel-

91 Associated Cells: VACs). In these cells a decrease in starch content and an increase in
92 sucrose concentration are observed upon refilling (Salleo et al. 2009; Secchi and
93 Zwieniecki 2010, Nardini et al. 2011). Sucrose is probably translocated to adjacent
94 embolised vessels (Holbrook and Zwieniecki 1999; Tyree et al. 1999; Salleo et al. 2004;
95 Secchi et al. 2012), where it helps to establish an osmotic gradient that draws water into
96 the emboli by aquaporin-mediated transport. The involvement of starch hydrolysis and
97 water transport facilitators in the refilling process is supported by upregulation of genes
98 encoding β-amylases and plasma membrane intrinsic proteins (PIPs) in recovering
99 shoots of *Juglans regia*, *Populus trichocarpa*, and *Vitis vinifera* (Sakr et al. 2003;
100 Kaldenhoff et al. 2008; Secchi and Zwieniecki 2010, 2011; Perrone et al. 2012b).
101 Besides describing the molecular processes involved, a few studies have focused on the
102 signal transduction pathways induced by the presence of xylem embolism. Secchi et al.
103 (2011) investigated global gene expression responses in poplar subjected to artificial
104 cavitation, and they proposed a novel role for oxygen as a signal molecule acting in
105 parenchyma cells and triggering xylem refilling. In previous studies (Lovisolo et al.
106 2008; Perrone et al. 2012b), we reported high levels of ABA in petioles recovering from
107 embolism under high transpiration conditions, and we hypothesized an active role of
108 this hormone in triggering recovery processes. Thus, the metabolic *scenario* of
109 embolism recovery is still debated. Moreover, although it is supposed that most of these
110 metabolic reactions take place in VACs, this has never been proven directly due to
111 technical difficulty of isolating these cells.
112 The Laser MicroDissection (LMD) technique is a powerful tool to isolate cell
113 populations from heterogeneous tissues and offers the possibility of exploring transcript
114 profiles in specific cell types. LMD has successfully been used to study gene expression
115 in different plant tissues, such as epidermal cells, shoot meristem tissues, root cap
116 tissues and specific cells involved in plant-microbe interactions, such as those colonised

by arbuscular mycorrhizal or pathogenic fungi (Balestrini et al. 2009; Gomez and Harrison 2009; Chandran et al. 2010; Giovannetti et al. 2012).

In this study, we induced xylem cavitation and subsequent recovery in grapevine leaf petioles using two different techniques: one involved pressure application and release to the stems, while the other consisted of water deprivation (drought) followed by irrigation. We used LMD to dissect VACs from embolised petioles, and we profiled the expression of genes involved in sugar metabolism and transport, as well as in water transport facilitation, in both VAC and whole petiole samples. We demonstrate that while some of the tested genes are activated by stress and subsequent recovery in whole petioles, some aquaporin genes are exclusively expressed in VACs, supporting the conclusion that the related proteins have a specific role in the embolism recovery process.

Materials and methods

Plant material and experimental setup

Two-year-old *Vitis vinifera* L. cv. Grenache plants [Vivai Cooperativi Rauscedo-San Giorgio della Richinvelda (PN), Italy] grafted onto *Vitis riparia* x *Vitis berlandieri* 420A were grown in a glasshouse under partially controlled climate conditions. The temperature in the greenhouse was maintained in the 26–35°C range, and natural light/night cycles were followed. Maximum photosynthetic photon flux density (PPFD) ranged between 1330 and 1580 μmol m^{-2} s^{-1}. Each plant grew in a 10-l pot filled with a substrate composed of a sandy-loam soil (pH 7.0; available P 7.9 mg kg^{-1}; organic matter 1.37%; cation exchange capacity 4.58 meq 100 g^{-1})/expanded clay/peat mixture

143 (2:1:1 by weight). From budbreak (February, 10th) to the beginning of the experimental
144 period (August 1), plants were irrigated twice a week to maintain water container
145 capacity.
146 Treatments were applied during a period of high atmospheric evaporative water demand
147 in August (vapour pressure deficit averaging 25 mbar bar^{-1}). Among the 36 plants used
148 in this study, 24 were maintained at container capacity (Lovisolo and Schubert 1998):
149 50% of these plants were used as control (12 IRR replicate plants), and 50% were
150 subjected to artificial cavitation, imposed using a pressure collar (PC) treatment (12
151 replicate plants) followed by depressurisation (RPC). The remaining 12 plants were
152 subjected to water stress (WS) treatment followed by rehydration (RWS).
153 Measurements and tissue samples were taken on one experimental day. To allow the
154 collection of data from a sufficient number of replicates, plants were distributed among
155 four experimental days: in each of them, three randomly chosen IRR, three PC-RPC,
156 and three WS-RWS plants were subjected to analysis.
157 For the PC treatment, shoots of normally irrigated plants were exposed to positive
158 pressures, following the procedure reported by several authors (Salleo et al. 1996, 2004;
159 Tyree et al. 1999; Secchi and Zwieniecki 2010) with minor modifications. Our system
160 consisted of a narrow-diameter tube (diameter, 19.1 mm) sealed around the basal
161 internode of the shoot by using a custom-built holder and allowing the application of
162 pressure around the stem. During the experimental day, at 11:00 h, the pressure collar
163 was connected to a gaseous N_2 bomb to maintain a 2.7 MPa pressure for five hours.
164 Thereafter (at 16:00 h), the collar was removed in order to induce depressurisation and
165 recovery.
166 For the WS treatment, irrigation was withheld for a 10-d period prior to the
167 experimental day. This treatment induces cavitation in grapevine without producing
168 stress-related modifications of xylem development (Schultz and Matthews 1988;

169 Lovisolo and Schubert 1998; Lovisolo et al. 2008). Water-stressed plants were
170 rehydrated at 16:00 h of the experimental day by watering pots to container capacity.
171 For each experimental day, one replicate plant within each treatment was used for: i)
172 leaf gas exchange and xylem embolism analysis; ii) leaf water potential measurement
173 and iii) petiole and leaf sampling for LMD, and for gene expression on whole petioles
174 and ABA analysis.
175
176 Leaf gas exchange, leaf water potential and xylem embolism
177
178 Transpiration rate (E) and stomatal conductance (g_s) were measured on adult, non-
179 senescing leaves well exposed to direct sunlight [PPFD (400–700 nm) ≥ 1200 μmol m^{-2}
180 s^{-1}], using an infrared gas analyser ADC-LCPro+ system (The Analytical Development
181 Company Ltd, Hoddesdon, UK). Measurements were taken on one leaf per plant at 30
182 min intervals between 10:00 and 19:00 h on each experimental day, and on IRR and
183 RWS plants also on the day after. Leaf water potential (Ψ_{leaf}) was assessed on one
184 transpiring leaf per plant and at each of the same time points by using a Scholander-type
185 pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA).
186 Xylem embolism extent was measured on leaf petioles, as previously described by
187 Lovisolo et al. (2008), using a Hydraulic Conductance Flow Meter (HCFM-XP,
188 Dynamax Inc., Houston, TX, USA) (Tyree et al. 1995). Measurements were made at
189 16:00 h of the experimental day for IRR, WS and PC treatments, at 19:00 h of the same
190 day for the RPC treatment, and at 19:00 h of the following day for the RWS treatment.
191 Briefly, one leaf petiole per plant was cut under water by bending the shoot and
192 submerging the petiole into a water container. Embolism extent was determined by
193 comparing the initial hydraulic conductivity (K_{hi}) with the maximum final hydraulic
194 conductivity (K_{hf}) recorded after a transient water flushing designed to eject the

195 embolism from the petiole. The intensity of embolism was expressed as the percentage
196 loss of conductivity (PLC) and calculated as 100 * (K_{hf} - K_{hi}) / K_{hf}. Significant
197 differences among treatments were determined by applying a one-way ANOVA test
198 using the SPSS statistical software package (SPSS Inc., Cary, NC, USA, v.20).
199
200 Laser microdissection of vessel-associated cells, RT-PCR and semi-quantitative RT-
201 PCR analyses
202
203 Two petioles per plant were collected at the same time points of PLC determination.
204 They were cut into about 5-mm segments and immediately fixed in Farmer's solution
205 (EAA), containing 75% (v/v) ethanol and 25% (v/v) acetic acid (Kerk et al. 2003), then
206 stored overnight at 4°C for paraffin embedding. Farmer's solution was then removed
207 and petiole segments were dehydrated in a graded series (30-min steps) of ice-cold
208 ethanol (70%, 90% in sterile water and 100% [v/v] twice), followed by 100% Neoclear
209 (Merck, Darmstadt, Germany). The petiole segments were then gradually replaced with
210 paraffin (Paraplast plus; Sigma-Aldrich, St Louis, MO, USA), following the protocol
211 described by Balestrini et al. (2007). Petiole sections (12 μm) were cut using a rotary
212 microtome and transferred onto Leica RNase-free PEN foil slides (Leica Microsystem,
213 Inc., Bensheim, Germany) with sterile double-distilled water (ddH_2O, Elga LabWater,
214 Lane End Industrial Park, UK). Sections were dried at 40°C in a warming plate, stored
215 at 4°C, and used within 1 day.
216 A Leica AS LMD system was used to isolate cells from dried sections. Just before use,
217 the paraffin sections were deparaffinised by Neoclear treatment for 10 min and 100%
218 ethanol for 1 min, and then they were air-dried. The slides were placed face down on
219 the microscope. Laser parameters for dissection of selected cells were 40-XT objective

at power 45–55, and speed 4. The cells from each biological replicate were subsequently collected (within one day) into a 0.5 ml RNase-free PCR tube.

After collection, 50 µl of PicoPure RNA extraction buffer (Arcturus Engineering, Mountain View, CA, USA) were added to each tube, followed by incubation at 42°C for 30 min, centrifugation at 800 *g* for 2 min, and storage at −80°C. RNA was extracted using the PicoPure kit (Arcturus Engineering). DNase treatment was not performed on the kit column, as described in the kit protocol, but RNA was treated with Turbo DNase after the extraction procedure (Applied Biosystems, Foster City, CA, USA), according with the manufacturer's instructions. RNA quality and quantity were checked using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A One-Step RT-PCR kit (Qiagen, Valencia, CA, USA) was used. Reactions were carried out in a final volume of 20 µl, as previously described by Balestrini et al. (2007). Samples were incubated at 50°C for 30 min, then at 95°C for 15 min. Amplification reactions were run for 40 cycles: 94°C for 45 s, 58°C for 45 s and 72°C for 45 s. All RT-PCR experiments were performed on at least two biological and two technical replicates. RNA samples were checked for DNA contamination through RT-PCR analyses conducted with the *VvEF1-α* specific primers. PCR products were separated on a 1.9% agarose gel. Target genes and relative primer pairs are described in Table S1.

Semi-quantitative RT-PCR experiments were carried out in a final volume of 21 µl following the same protocol. Amplification reactions with specific primers for the selected genes (Table S1) and control gene (*VvEF1-α*) were run for different cycles (35, 37, 40) to determine the exponential amplification phase, as previously reported by Guether et al. (2009). For each step of semi-quantitative RT-PCR, 7 µl of cDNA were loaded on a 1.9% agarose gel.

246 Quantitative expression analysis on whole petioles and leaf ABA concentration

247

248 Expression changes of target transcripts were quantified on whole petiole samples by
249 quantitative real-time PCR (RT-qPCR). Two leaves per plant were collected at the same
250 time points of PLC determination. Petioles from each treatment were pooled,
251 immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted in
252 triplicate from the pooled samples following the protocol by Carra et al. (2007). RNA
253 integrity and quantity were checked using a 2100 Bioanalyser (Agilent, Santa Clara,
254 CA, USA). RNA samples were treated with DNase I, RNase-free (Fermentas: 50 U μl^{-1}),
255 and first-strand cDNA was synthesised starting from 10 µg of total RNA by using the
256 High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the
257 manufacturers' instructions.
258 Reactions were carried out in a StepOnePlus™ RT-qPCR System (Applied Biosystems)
259 and the SYBR Green method (Power SYBR® Green PCR Master Mix, Applied
260 Biosystems) was used for quantifying amplification results (Perrone et al. 2012a). Three
261 technical replicates were run for each sample. Thermal cycling conditions were as
262 follows: an initial denaturation phase at 95 °C for 10 min, followed by 40 cycles at 95
263 °C for 15 s and 60°C for 1 min (only for aquaporin primers a step at 56°C for 15 s was
264 added to the cycling stage). Specific annealing of primers was checked on dissociation
265 kinetics performed at the end of each RT-qPCR run. Expression of target transcripts
266 was quantified after normalisation to the geometric mean of the endogenous control
267 genes, *Ubiquitin* (*VvUBI*) and *Actin* (*VvACT1*). Gene expression data were calculated as
268 expression ratios (relative quantity, RQ) to IRR controls. Gene-specific primers are
269 listed in Table S1. Significant differences between treated and control samples were
270 investigated by applying a one-way ANOVA test ($P < 0.05$), using the SPSS statistical
271 software package (SPSS, v.20).

272 Leaf blades for ABA analysis were also immediately frozen at −80°C, and ABA
273 concentration was quantified following the method previously described by Lovisolo et
274 al. (2008).
275
276

Results

Leaf physiological parameters and leaf ABA concentration

Stem pressurisation obtained by application of a pressure collar induces xylem embolism formation without imposing long-lasting stress on the organs located distally to the collar. We compared the physiological responses (leaf gas exchange, petiole xylem embolism, and petiole ABA concentration) induced by application of either the pressure collar or water stress, and by the following depressurisation or rehydration.

As expected, application of the pressure collar effectively induced xylem embolism. percentage loss of hydraulic conductivity (PLC) was about 10% in IRR petioles. WS treatment induced an increase in PLC to about 80%, and application of the PC also induced an increase in PLC to about 60%. Embolism recovery proceeded much faster in RPC than in RWS petioles: upon irrigation of WS plants, PLC only decreased to 54% after 27 h from the re-watering treatment, while stem depressurisation (RPC) allowed almost full recovery from embolism within 3 h (10% PLC) (Fig. 1).

Application of the PC also induced water stress in the leaves, as shown by measurements of leaf water potential (Ψ_{leaf}) and stomatal conductance (g_s), albeit at a lower level than the application of WS. In irrigated (IRR) petioles, Ψ_{leaf} remained relatively constant between −0.3 and −0.4 MPa. Ψ_{leaf} decreased to about −1.4 MPa upon WS, and to about −1.2 MPa upon PC application (Fig. 2a). In IRR plants g_s averaged 0.14 mol m^{-2} s^{-1} with an expected slight decrease in the afternoon. Leaf g_s was lower in WS plants (less than 0.03 mol m^{-2} s^{-1}) and in PC plants 0.06 mol m^{-2} s^{-1} (Fig. 2b). Leaf transpiration rate (E) reflected the observed changes in g_s (Fig. 2c).

The behaviour of plants subjected to WS versus PC treatment was, however, quite different when a recovery was induced by rehydration in the case of WS (RWS), and by

depressurisation in the case of PC (RPC). In RWS petioles, Ψ_{leaf} recovered very slowly, reaching -0.5 MPa at 19:00 h the following day, while recovery upon depressurisation was fast and complete, reaching −0.5 MPa at 19:00 h the following day, while recovery upon depressurisation was fast and complete, reaching −0.5 MPa within 3 h (Fig. 2a). Also, g_s and E recovery were slow in RWS plants, reaching about 50% of that of the IRR controls at 19:00 h the following day, while, in RPC plants, g_s quickly (within 3 h) recovered (Fig. 2b,c).

Since the maintenance of responses to water stress after rehydration in grapevine is dependent on the persistence of stress-induced endogenous ABA, we reasoned that the physiological differences observed between WS-RWS and PC-RPC plants could be due to different intensities of an ABA signal. The ABA concentration in leaves of irrigated plants did not differ from that measured in PC and RPC leaf samples, while in WS leaf ABA was significantly higher, with values around 13 000 pmol g^{-1} DW. At the end of recovery from water stress (RWS), ABA levels dropped to values comparable with those of the IRR controls (Fig. 3).

Laser microdissection and analysis of gene expression in vessel-associated cells and whole petioles

The LMD protocol preserved petiole vascular tissues fairly well, which allowed identification of specific cell types, in particular VACs (present among xylem vessels) and phloem (Fig. 4a,b). For each treatment, about 270–300 vascular cell groups were obtained. RNA final concentrations ranged between 10 and 30 ng μl^{-1}, depending on the sample type and on the number of collected vascular cell groups. RNA samples from the LMD-isolated tissue were then used to study the expression of genes putatively involved in xylem embolism formation and repair. More specifically, all

target transcripts were first analysed in VACs by carrying out one-step RT-PCR experiments. In RT-plus reactions the presence of an amplified fragment of the expected size (100 bp) was observed in all cell types tested, using specific primers for the endogenous control gene *VvEF1-α*, while the absence of an amplified product in RT-minus reactions excluded genomic DNA contamination (Fig. S1). Since for the majority of the genes an expression signal was observed in several of the considered treatments (data not shown), we further investigated transcript expression by semi-quantitative PCR analysis after 35, 37 and 40 amplification cycles (Fig. 4). After 40 cycles, the amplification had reached its plateau in all samples, whereas after 35 and 37 cycles it was still in the exponential phase, thus allowing a semi-quantitative comparison of transcript abundance. As shown in Fig. 4c, transcript abundance of the control gene *VvEF1-α* was comparable in all samples. We analysed the expression of 12 genes related to drought and ABA responses to sugar metabolism and to water transport, which are regulated in grapevine petioles upon water stress and rehydration (Perrone et al. 2012b). More specifically, we considered: three genes involved in sugar metabolism—a plastidic glucose-6P transporter (*VvGPT1*), a sucrose transporter (*VvSUC27*), and a plastidic β-amylase (*VvBAM3*); two genes encoding proteins belonging to the LEA (Late Embryogenesis Abundant) family (*VvDHN1a* and *VvLEA14*); three genes encoding components of signal transduction (*VvNAC72, VvSnRK2.1,* and *VvCAL*) and activated by drought and ABA in several systems; and four genes encoding PIP-type aquaporins—namely *VvPIP1;1*, *VvPIP1;2*, *VvPIP2;1* (Vandeleur et al. 2009) and *VvPIP2;4N* (Perrone et al. 2012a). Expression analyses performed on micro-dissected VACs showed that *VvGPT1* was activated by PC and RPC treatments; *VvSUC27* expression was low in all treatments, while *VvBAM3* was activated in WS- and PC-treated cells (Fig. 4d,f). Results on genes potentially tied to signal transduction mechanisms showed that *VvSnRK2.1* was activated in WS petioles,

while *VvNAC72*, besides in WS cells, was also activated in PC and RPC samples (Fig. 4g,h); *VvCAL* transcripts were only detected in WS and RWS cells (Fig. 4k). Among aquaporin genes, *VvPIP1;1* was one of the most highly expressed in embolism-inducing treatments (WS and PC), but it was also activated in RWS and RPC cells (Fig. 4i). *VvPIP1;2* expression was undetectable in all treatments (data not shown). *VvPIP2;1* was more expressed in PC and RPC treatments than in the other treatments (Fig. 4j); however, *VvPIP2;4N* transcripts were only observed in WS and mostly in RWS cells (Fig. 4l). Considering the members of the LEA family, which are typically involved in plant stress response, *VvDHN1a* was exclusively expressed in WS and PC cell samples, whereas *VvLEA14* was mainly activated in PC and in RPC cells and to a lesser extent in WS cells (Fig. 4m,n).

To verify the specificity of gene expression in VACs, quantitative real-time PCR (RT-qPCR) experiments were performed on the same target genes working on whole petiole samples. *VvGPT1* and *VvSUC27* expression followed the same patterns observed in VACs (Fig. 5a,b). *VvBAM3* was significantly activated upon WS treatment (as in VACs), but it was down-regulated in PC and RPC petioles, despite these latter expression changes were not significant when compared to the IRR control (Fig. 5c). The two *LEA* genes were both strongly up-regulated upon WS treatment and their expression was still very high in RWS samples (Fig. 5d,e). Moreover, *VvLEA14* was significantly over-expressed in PC petioles, mirroring the pattern observed in VACs (Fig. 5d). No significant changes were observed for *VvDHN1a* transcripts in PC samples compared to the IRR control (Fig. 5e), while the same gene was activated in VACs upon this treatment.

In whole petiole, the expression of genes encoding components of signal transduction followed patterns similar to those observed in VACs: both *VvNAC72* and *VvSnRK2;1* levels increased in WS and RWS treatments (Fig. 5f,g), although the over-expression of

VvSnRK2;1 was significant only in WS samples (Fig. 5g). Interestingly, *VvNAC72* was also slightly activated in PC and RPC treatments (Fig. 5f), whereas *VvSnRK2;1*transcripts underwent a significant down-regulation.

The same consideration can be made for *VvCAL* transcripts, which were highly expressed in WS ~~and RWS~~ petioles, following the expression profile observed in VACs, while they were significantly down-regulated in both PC and RPC samples (Fig. 5h).

Among aquaporin genes, *VvPIP1;1* was slightly activated in WS petioles and significantly down-regulated in RWS, PC and RPC petioles (Fig. 6a), at variance with the observations made in VACs; *VvPIP1;2* was up-regulated in all treatments compared to the IRR control (Fig. 6b), whereas in all VAC samples the same gene was not detected.

Finally, while the expression of *VvPIP2;1* followed the same pattern observed in VACs (it strongly increased in PC and RPC), *VvPIP2;4N* transcriptional levels were significantly down-regulated in all treatments (Fig. 6c,d).

Discussion

Induction of xylem embolism in grapevine by water stress and stem pressurisation

It is well known that water stress (and subsequent rehydration) can induce xylem embolism formation and recovery. Nevertheless, this environmental condition triggers a wide array of molecular changes, which can mask those strictly related to embolism refilling processes. In order to control these masking effects, we employed, parallel to water stress and rehydration, the technique of stem pressurisation/depressurisation to

induce embolism formation and repair with a limited incidence of other stress-induced processes. Both water stress and stem pressurisation require petiole excision to assess the degree of embolism, and this was reported to induce artefacts on *Acer rubrum* and *Fraxinus americana* (Wheeler et al. 2013). However, direct observations of embolism recovery obtained in the absence of petiole excision (Brodersen et al. 2010, 2013) suggest that refilling in grapevine is not affected by such artefacts (Sperry, 2013).

In our experiment, stem pressurisation was obtained by applying a pressure collar to grape stems. Other authors have already used artificial tools to induce xylem cavitation in woody plants (e.g., Salleo et al. 1996; Mayr et al. 2006; Secchi and Zwieniecki 2011). These systems are particularly suited to increasing pressure gradients at air-water interfaces into the plant organs, thus inducing embolism formation. The method we set up avoids both air injection bores and wounding to create the air inlet. After about five hours of PC treatment, PLC increased from 10% to 55% in petioles; following depressurisation, PLC fully recovered within 3 hours.

The PC treatment was not devoid of effects on water potential and leaf gas exchange, which decreased after pressurisation and recovered upon depressurisation. Nevertheless, the time courses of PLC, water potential, and leaf gas exchange upon RPC and RWS were clearly different, since RPC recovery kinetics were more rapid. Depressurised twigs of laurel, previously submitted to a pressure collar treatment, showed faster and larger xylem refilling than upon native embolism repair (Salleo et al. 1996). A slow recovery of hydraulic conductance and transpiration after rehydration of drought-exposed plants has been well documented in grapevine and linked to the persistence of high ABA concentration after rehydration (Lovisolo et al. 2008; Flexas et al. 2009; Zufferey et al. 2011; Perrone et al. 2012b). ‘Grenache’ is a drought-avoiding isohydric grape genotype, particularly suited to study drought responses, since it is able to tolerate long-term water stress conditions (Schultz 2003; Soar et al. 2004; Vandeleur et al. 2009)

through ABA-mediated control of stomatal closure (Lovisolo et al. 2008, 2010). In this experiment, recovery in RPC plants was indeed associated with low leaf ABA concentration.

Differences in leaf ABA concentration, and in the kinetics and intensity of leaf water potential and gas exchange changes induced by the two types of treatment, likely reflect diverse mechanisms of induction of xylem embolism. In the case of drought-induced water stress, water status is negatively affected, and ABA concentration increases in the leaves, inducing stomatal closure: increased PLC is thought to depend on the increased xylem tension that develops as an effect of water potential changes. In the case of stem pressurisation, no water loss takes place, and xylem embolism is likely the primary effect, later followed by limitations of leaf water potential due to reduced xylem hydraulic conductivity, and by stomatal closure. In this case, a reversible loss of leaf hydraulic conductivity could be a means of amplifying the signal of evaporative demand to the stomata in order to trigger the stomatal response, as suggested by Brodribb and Holbrook (2004) and shown in grapevine by Zufferey et al. (2011).

Expression changes of genes putatively involved in embolism recovery

Embolism recovery is an active process, which requires energy and metabolic activity. It takes place upon negative tensions in the xylem, and several mechanistic models have been proposed to explain it. All these models converge in considering the pivotal role of solutes, solute transporters, and water transport facilitators (aquaporins) in VACs. Recently, two studies based on transcriptomic analysis of tissues undergoing embolism recovery have reported some genes linked to these processes, which undergo significant expression changes (Secchi et al. 2011; Perrone et al. 2012b). Nevertheless, these studies have focused on whole tissues, where molecular processes localised in VACs

459 may not be detectable. To deepen the role of these genes, we have analysed the
460 expression of some of these transcripts in VACs isolated by LMD.
461 The importance of regulation of carbohydrate metabolism and transport in VACs during
462 the embolism recovery process has already been supported by physiological analyses
463 (Salleo et al. 1996; Nardini et al. 2011; Secchi et al. 2013) and by measurements of gene
464 expression changes (Secchi et al. 2011; Perrone et al. 2012b). *VvBAM3* encodes a beta-
465 amylase up-regulated by water stress (Perrone et al. 2012b) as its *Arabidopsis*
466 orthologue (Fulton et al. 2008). *VvGPT1* is annotated as a plastidic glucose-6P
467 symporter and is up-regulated upon embolism recovery (Perrone et al. 2012b). Its
468 closest *Arabidopsis* homologue, the Glc6P/phosphate translocator1 (*At*GPT1), is
469 localised in vascular bundle sheath cells (Niewiadomski et al. 2005), where it
470 contributes to glucose-6-phosphate transport into plastids (Kunz et al. 2010). In grape
471 petioles, *Vv*GPT1 could operate in the reverse direction, providing a supply of GLU-6P
472 into the cytosol of VACs. *VvGPT1* and *VvBAM3* genes were both activated in VACs
473 upon the embolism-inducing treatments applied (WS and PC). This data is in agreement
474 with a picture of activated starch hydrolysis and GLU-6P export from plastids, which
475 provides soluble sugars required to support the embolism recovery process. *Vv*SUC27 is
476 an H^+-dependent sucrose transporter, whose expression is associated with sink organs in
477 grape (Davies et al. 1999). In whole grape petioles, *VvSUC27* is down-regulated by all
478 treatments inducing embolism formation, and it has previously been observed to be also
479 down-regulated due to water stress (Perrone et al. 2012b). This suggests that, upon
480 xylem embolism, the main provision of sugars to VACs derives from starch breakdown
481 and not from phloem unloading. *VvSUC27* expression was almost absent in VACs,
482 where phloem cells are not present. However, the regulatory changes involving these
483 genes were not limited to VACs. Indeed, they were also detected in whole petioles,

484 suggesting that most of the petiole cells collaborate each other to the mobilisation of
485 soluble sugars that drives embolism recovery.
486 The picture was quite different in the case of aquaporins, which are thought to facilitate
487 water supply to the xylem, thus determining a successful refilling process (Kaldenhoff
488 et al. 2008). This hypothesis requires that the activation of these channels takes place in
489 the cells surrounding xylem vessels. Among the tested *PIP1* and *PIP2* genes, *VvPIP1;1*,
490 *VvPIP2;1* and *VvPIP2;4N* were expressed in VACs of either embolising or recovering
491 petioles, confirming a potential role for these proteins in embolism refilling. However,
492 in the case of *VvPIP1;1* and *VvPIP2;4N* genes, these expression differences were not
493 observed in whole petioles, both in this study and in a previous work by Perrone et al.
494 (2012b), suggesting that their activation was strictly localised in VACs. The role of
495 aquaporins in embolism refilling has been inferred from expression measurements
496 performed in different plants, such as olive (Secchi et al. 2007), grapevine (Galmés et
497 al. 2007), rice (Sakurai-Ishikawa et al. 2011), tobacco (Mahdieh et al. 2008), and poplar
498 (Secchi et al. 2011). Aquaporins could contribute to embolism refilling only indirectly,
499 by facilitating axial flow of water to the leaves and thus reducing the xylem tension
500 gradient. Our expression results, obtained for the first time at the VAC level, strengthen
501 the hypothesis that these aquaporins play a pivotal role in refilling xylem embolism.
502 On the contrary, *VvPIP2;1* follows a different model. Indeed, the activation of this gene
503 takes place both in VACs and whole petioles, suggesting that it is probably not directly
504 linked to either embolism formation or recovery, but it could indirectly contribute to the
505 process. Finally, *VvPIP1;2* was activated in petioles but not in VACs, and this points to
506 a dependency on stress but not to a role in embolism refilling.
507
508 Water-stress and pressure collar responses to xylem embolism
509

In PC plants, embolism induction and recovery were faster than in WS plants, and they took place in the absence of an ABA confounding effect. Since ABA strictly controls gene expression networks in plants and grapevine (Koyama et al. 2009), we thus checked whether expression of genes induced by water stress and of genes involved in embolism recovery could be affected by the two different treatments.

Two genes belonging to the late embryogenesis abundant (LEA) protein family, encoding a LEA14 (*VvLEA14*) and a dehydrin (*VvDHN1a*), were tested. In *Arabidopsis*, the *VvDHN1a* orthologue (AT1G07470) is activated by salt and cold stress, and by ABA (Hundertmark et al. 2008). In grapevine, *VvDHN1a* expression is induced by water stress (Cramer et al. 2007) and ABA (Koyama et al. 2009; Yang et al. 2012). Our results show that both genes are activated upon WS in VACs and whole petiole samples, as previously observed in cv. Cabernet Sauvignon by Cramer et al. (2007). Nevertheless, in VACs these genes were also up-regulated upon PC treatment.

We further measured the expression of three stress-responsive genes involved in signal transduction (*VvCAL, VvSnRK2;1, VvNAC72*). In detail, *VvCAL* is the grape orthologue of the *AtCLM24* (AT5G37770) gene, which encodes a Ca^{2+} binding protein in response to ABA stimulus, day length and salt stress (Delk et al. 2005). *VvSnRK2;1* encodes a protein kinase involved in ABA signal transduction, strongly up-regulated in grape leaves treated with exogenous ABA (Boneh et al. 2012). *VvNAC72* is the grape orthologue of *AtNAC72* (AT4G27410), whose expression is strictly controlled by ABA (Fujita et al. 2004); in grape petioles this gene is activated upon water stress (Perrone et al. 2012b). Our results indicate that *VvCAL* is only activated in WS and RWS VACs, and in WS whole petioles; *VvSnRK2;1* transcripts are more abundant upon WS both in VACs and whole petioles, although a slight up-regulation of this gene could be observed in VACs upon PC, RPC and RWS treatments. On the contrary, *VvNAC72* is more expressed in PC and RPC VACs.

Such differences between the two treatments were also observed for genes putatively related to embolism recovery. Expression of *VvPIP2;4N*, and, to a lesser extent of *VvBAM3*, increased upon water stress. *VvPIP2;4N* is a root-specific grape aquaporin (Perrone et al. 2012a) and the localisation of its expression in VACs, depending on water stress, could explain the fact that this transcript is not detected in whole petioles. In olive twigs it has been shown that *OePIP2;1* aquaporin expression is activated when shoot hydraulic conductance recovers (Secchi et al. 2007), and generally there is an up-regulation of aquaporin genes when rehydration also occurs in grapevine leaves (Galmés et al. 2007) or petioles (Perrone et al. 2012b). In addition, a coupling of aquaporin activation with an increment in leaf transpiration has also been reported in rice roots, where transpiration demand triggers the up-regulation of PIPs localised both at the proximal end of the endodermis and on the cell surface around xylem (Sakurai-Ishikawa et al. 2011), and in drought-exposed/rehydrated tobacco roots (Mahdieh et al. 2008). An obvious candidate for gene activation exclusively under drought stress is a surge in ABA concentration, and correspondingly we found no ABA increase in PC and RPC-treated leaves. We have previously shown (Lovisolo et al. 2008; Perrone et al. 2012b) that, upon rehydration from water stress, grapevine leaves accumulate ABA at levels even higher than during the stress itself, and this could be instrumental to embolism recovery if contemporaneously VAC-specific aquaporins are activated as is the case of *Vv*PIP2;4N.

Other genes (*VvGPT1* and *VvPIP2;1*) are present only upon pressure collar pressurisation and depressurisation. These treatments thus trigger embolism-induced signals that are not induced in water-stressed plants, although embolism is also present in the latter. An explanation for this apparently contradictory result can be found in the different dynamics of embolism induction and recovery deriving from the two types of treatment. These dynamics are much faster in PC and RPC treatments. This means that,

562 during PC and RPC treatments, a fast induction of embolism could elicit signals that are
563 not present when a slow induction of embolism occurs, such as the case of water stress
564 treatment. Secchi and Zwieniecki (2010), also using an artificial device to induce
565 formation of xylem embolism in poplar, proposed several possible signals evoked
566 during fast embolism induction, such as the accumulation of soluble sugars in the xylem
567 or oxidative stress. However, in natural (and agricultural) conditions, xylem embolism
568 almost invariably arises because of drought. The experimental use of devices, such as
569 the pressure collar, which is applied to obtain embolism in the absence of water stress,
570 could not be representative of this condition, since this condition seems to activate
571 genes that are not expressed by water stress and following recovery.
572
573
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579

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774 **Figure captions**

775

776 **Fig. 1** Percentage loss of hydraulic conductivity (PLC) measured on cv. Grenache
777 petioles. IRR, irrigated control; WS, water stress; RWS, recovery from water stress; PC,
778 pressure collar stress; RPC, recovery from pressure collar stress. Lower case letters
779 denote significant differences ($P < 0.05$) attested by using the Tukey's test, bars are
780 standard errors of the mean ($n = 4$)

781

782 **Fig. 2** Time course of daily changes in leaf water potential (Ψ_{leaf}, **a**), stomatal
783 conductance (g_s, **b**), and leaf transpiration (E, **c**), measured on cv. Grenache plants well
784 watered (IRR), subjected to water stress (WS) and pressure collar (PC) treatments. Gray
785 arrow displays the time of PC pressurisation and black arrow shows the time of both
786 WS re-watering and PC depressurisation, as described in Materials and Methods. Bars
787 are standard errors of the mean ($n = 4$). Boxes containing initials are positioned
788 according with the sampling time. IRR, irrigated control; WS, water stress; RWS,
789 recovery from water stress; PC, pressure collar stress; RPC, recovery from pressure
790 collar stress

791

792 **Fig. 3** ABA concentration (pmol g^{-1} DW) in leaves of cv. Grenache plants. Lower case
793 letters denote significant differences ($P < 0.05$) attested by using the Tukey's test; bars
794 are standard errors of the mean ($n = 4$)

795

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Fig. 4 Microdissection of vessel associated cells around xylem vessels (target section area is indicated with a black line) before (**a**) and after (**b**) laser cutting. The inset shows collected cells; red arrows indicate vessel-associated cells. Pictures were taken using an x40 objective lens; scale bars represent 50 µm. Xyl: xylem cells; Phl: phloem cells. **c-j** Semi-quantitative RT-PCR analyses on micro-dissected cells using the elongation factor gene (*VvEF1-α*) (**c**) as endogenous control. Numbers correspond to RT-PCR cycles. **k-n** RT-PCR on micro-dissected cells using specific primers for *VvCAL* (**k**), *VvPIP2;4N* (**l**), *VvDHN1a* (**m**) and *VvLEA14* (**n**) genes. The size of amplified sequences is 100 bp. IRR, irrigated control; WS, water stress; RWS, recovery from water stress; PC, pressure collar stress; RPC, recovery from pressure collar stress

Fig. 5 Expression analysis of target genes in whole petioles: **a-h** RT-qPCR analyses on cv. Grenache petioles for *VvGPT1* (**a**), *VvSUC27* (**b**), *VvBAM3* (**c**), *VvLEA14* (**d**), *VvDHN1a* (**e**), *VvNAC72* (**f**), *VvSnRK2;1* (**g**) and *VvCAL* (**h**) transcripts. *Ubiquitin* (*VvUBI*) and *Actin1* (*VvACT1*) were used as endogenous control genes for the normalisation procedure. IRR, irrigated control; WS, water stress; RWS, recovery from water stress; PC, pressure collar stress; RPC, recovery from pressure collar stress. Lower case letters denote significant differences ($P < 0.05$) attested by using the Tukey's test, bars are standard errors of the mean ($n = 3$).

Fig. 6 Expression analysis of target aquaporin genes in whole petioles: **a-d** RT-qPCR analyses on cv. Grenache petioles for *VvPIP1;1* (**a**), *VvPIP1;2* (**b**), *VvPIP2;1* (**c**) and *VvPIP2;4N* (**d**) transcripts. *Ubiquitin* (*VvUBI*) and *Actin1* (*VvACT1*) were used as endogenous control genes for the normalisation procedure. IRR, irrigated control; WS, water stress; RWS, recovery from water stress; PC, pressure collar stress; RPC,

822 recovery from pressure collar stress. Lower case letters denote significant differences (P
823 < 0.05) attested by using the Tukey's test, bars are standard errors of the mean ($n = 3$).
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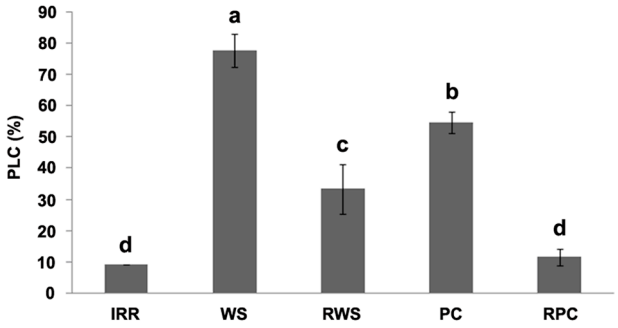

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828 Fig 1

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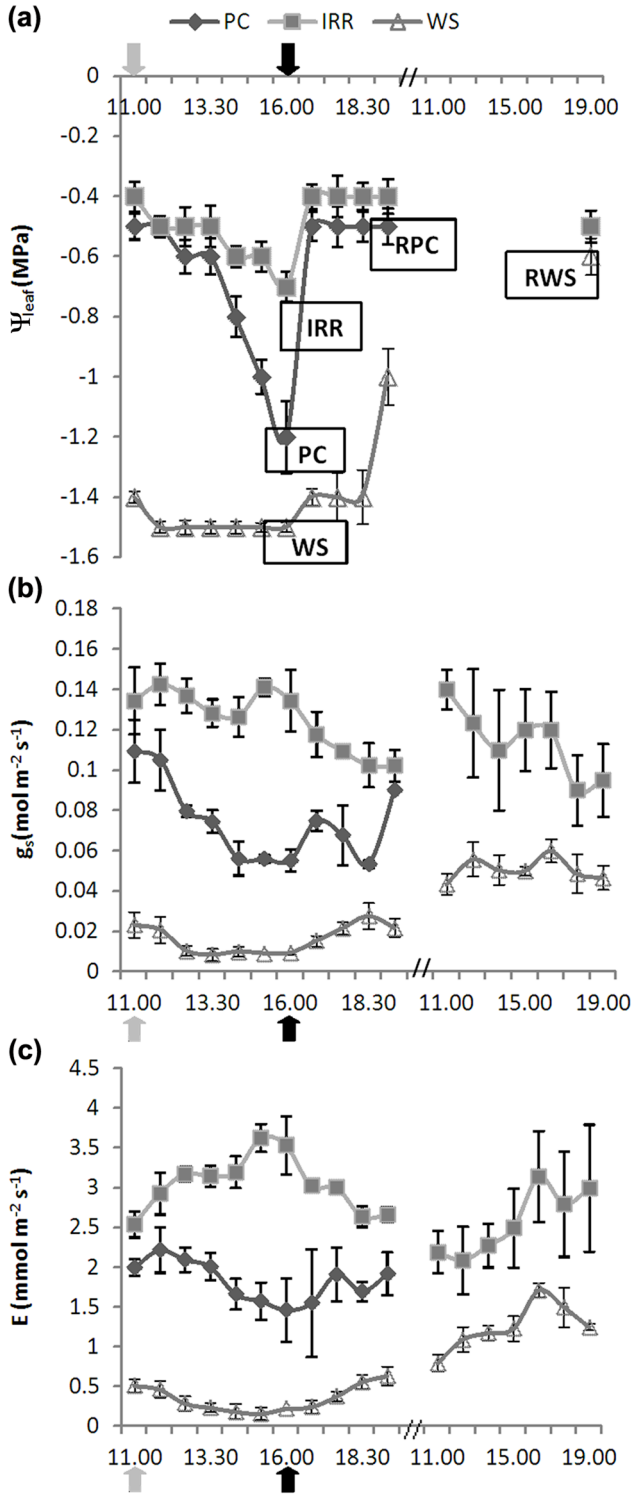

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831 Fig 2

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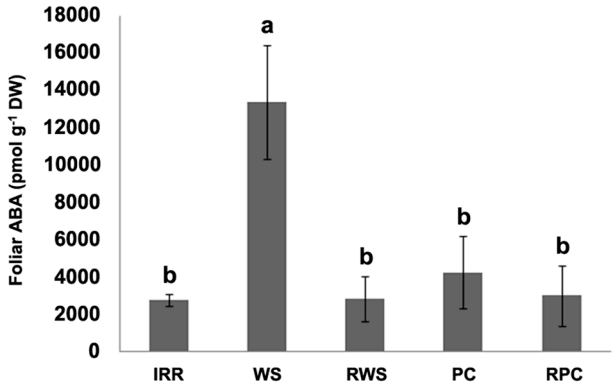
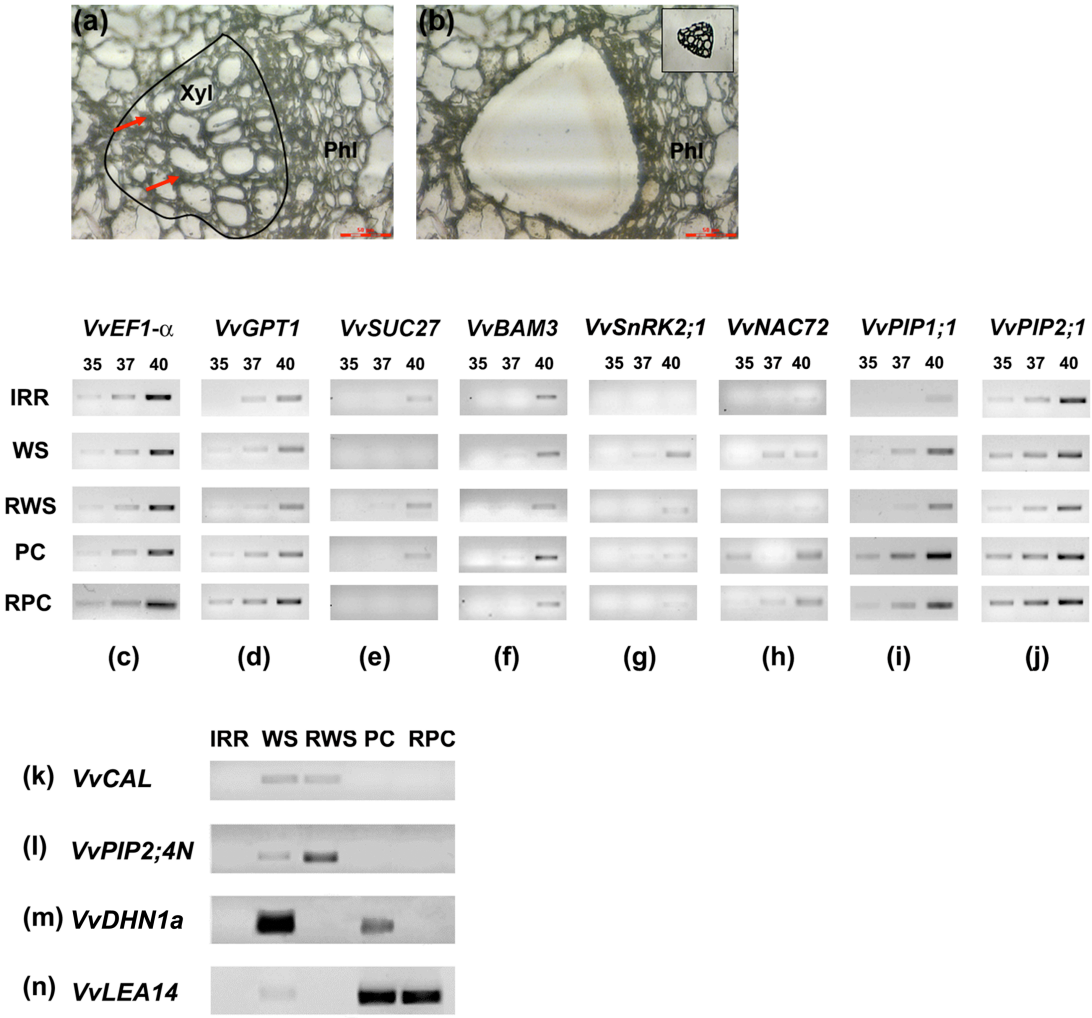

18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Foliar ABA (pmol g-1 DW)
a
b
b
b
b
IRR
WS
RWS
PC
RPC

Fig 3



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840 Fig 4

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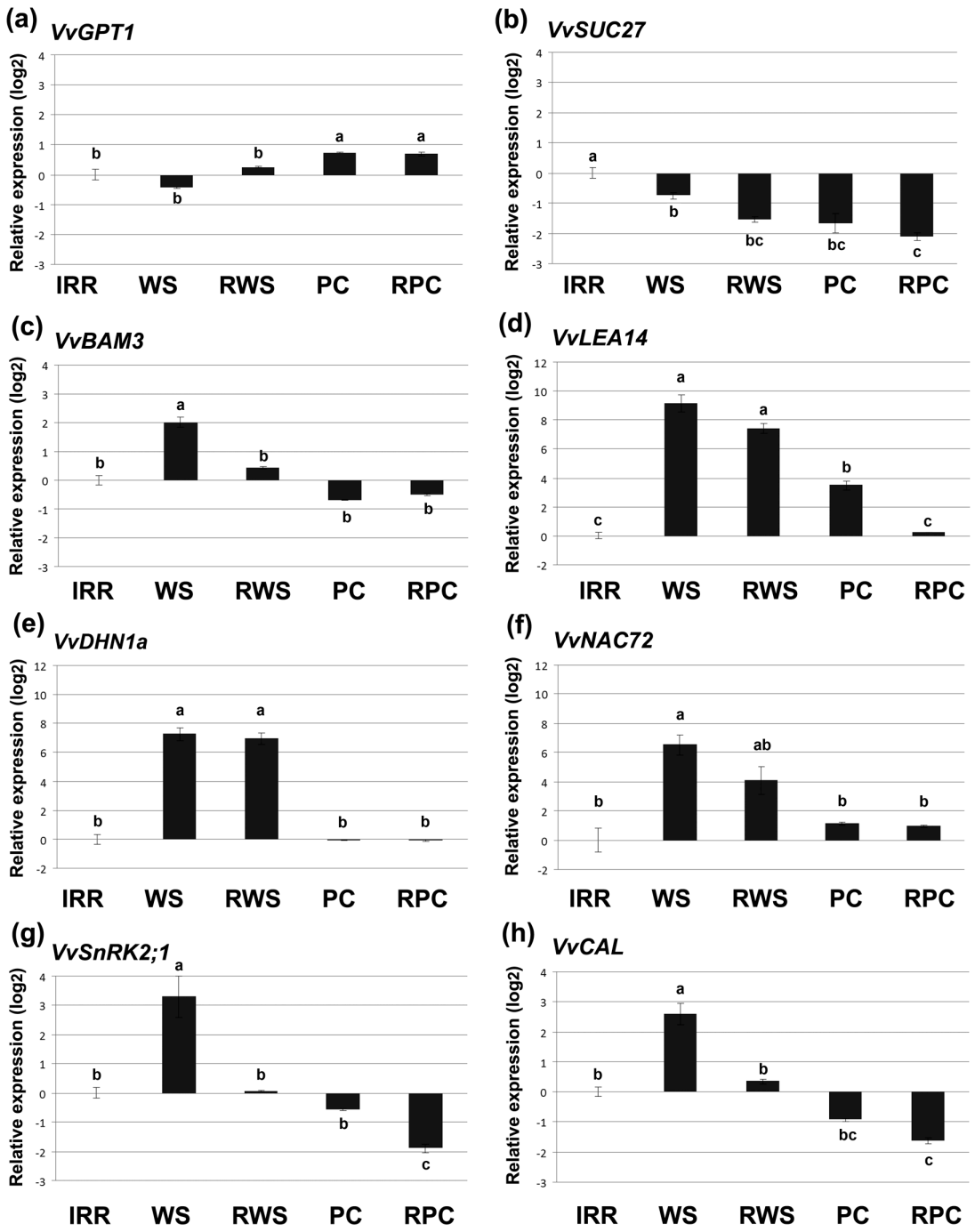

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843 Fig 5

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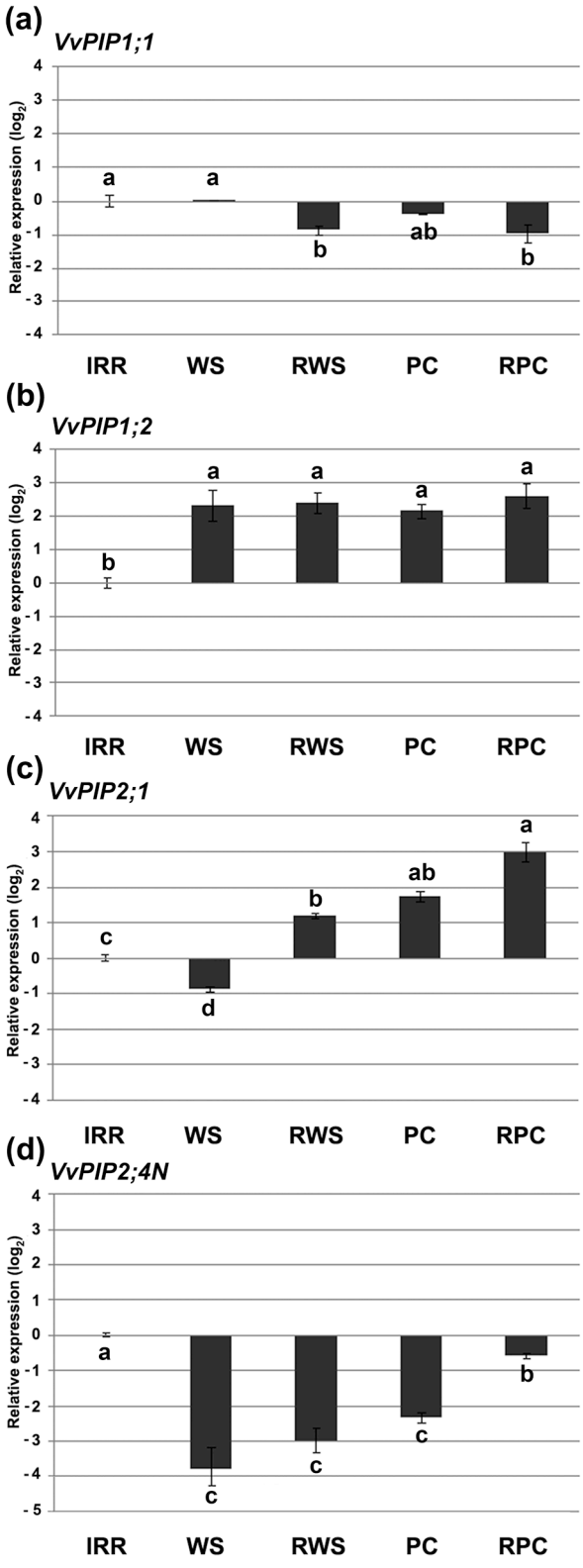
(a)
VvPIP1;1
Relative expression (log2)
4
3
2
1
0
-1
-2
-3
-4
a
a
b
ab
b
IRR
WS
RWS
PC
RPC
(b)
VvPIP1;2
Relative expression (log2)
b
a
a
a
a
IRR
WS
RWS
PC
RPC
(c)
VvPIP2;1
Relative expression (log2)
c
d
b
ab
a
IRR
WS
RWS
PC
RPC
(d)
VvPIP2;4N
Relative expression (log2)
5
a
c
c
c
b
IRR
WS
RWS
PC
RPC

Fig 6

Supplementary material captions

Supplementary Table S1 Oligonucleotides used in RT-PCR and semi-quantitative RT-PCR on LMD samples, and in RT-qPCR on whole petiole samples

Supplementary Fig. S1 RT-PCR analysis on micro-dissected samples using primers for *VvEF1-α* gene as endogenous control gene. Any amplified product in RT-reactions excludes DNA contaminations. IRR, irrigated control; WS, water stress; RWS, recovery from water stress; PC, pressure collar stress; RPC, recovery from pressure collar stress

860 **Supplementary material**

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862 **Table S1** Oligonucleotides used in RT-PCR and semi-quantitative RT-PCR on LMD samples, and

863 in RT-qPCR on whole petiole samples

Gene description	Gene ID (VVGDB 12X) and References	Primer	Primer sequences 5'-3'
Actin 1 (VvACT1)	VIT_04s0044g00580	Forward	GCCCCTCGTCTGTGACAATG
	Perrone *et al.*, 2012b	Reverse	CCTTGGCCGACCCACAATA
Ubiquitin (VvUBI)	VIT_16s0098g01190	Forward	TCTGAGGCTTCGTGGTGGTA
	Perrone *et al.*, 2012b	Reverse	AGGCGTGCATAACATTTGCG
Elongation factor 1-alpha	VIT_06s0004g03240	Forward	GAACTGGGTGCTTGATAGGC
(VvEF1-α)	Reid *et al.*, 2006	Reverse	AACCAAAATATCCGGAGTAAAAGA
Dehydrin 1a (VvDHN1a)	VIT_04s0023g02480	Forward	AACCCGGCGTGCTTCAT
	Perrone *et al.*, 2012b	Reverse	CATGCCCGGTATCCTCTCTTT
Late Embryogenesis Abundant Protein 14	VIT_15s0046g02110	Forward	CGTACAACGCCAAGGTCTCA
(VvLEA14)	Perrone *et al.*, 2012b	Reverse	CATCTTCCCCGACGCTATCA
NAC domain-containing protein 72-like	VIT_19s0014g03290	Forward	CGCCCTCCAATCTTCTTCTCT
(VvNAC72)	Perrone *et al.*, 2012b	Reverse	AGCTGTGAAAGCGGGTCAGT
Serine threonine kinase 2.1	VIT_02s0236g00130	Forward	AGATGTTTGGTCTTGTGGTGTGA
(VvSnRK2;1)	Perrone *et al.*, 2012b	Reverse	CCCAATGGTCTTCCGGAAAT
Calmodulin-like protein (VvCAL)	VIT_15s0048g00790	Forward	TGGTCAGAGAAGTGGACTGCAA
	Perrone *et al.*, 2012b	Reverse	CAGGTGCTGCTGCTACCAACT
Beta amylase (VvBAM3)	VIT_02s0012g00170	Forward	CTAGCAGCTGCCGAAGGAAT
	Perrone *et al.*, 2012b	Reverse	CAGCCGCATGAGACCTTGTT
Glucose-6-phosphate transporter	VIT_10s0116g00760	Forward	TTCCGGTGCCGGTCTACTT

(VvGPT1)	Perrone *et al.*, 2012b	Reverse	GCCCCCATAAACCCAGTCAT
Sucrose transporter (VvSUC27)	VIT_18s0076g00250	Forward	TGACCCCCTACGTTCAGCTT
	Perrone *et al.*, 2012b	Reverse	CCAACTACCGGCTGCACAAT
Aquaporin PIP1;1 (VvPIP 1;1)	VIT_13s0067g00220	Forward	GAGTGGTGCTGGGCGTTGATC
	Choat *et al.*, 2009	Reverse	GTGGAATGCTACAGACATTAC
Aquaporin PIP1;2 (VvPIP 1;2)	VIT_15s0046g02420	Forward	TCCTCCATTTTCGTTTCTTC
	Choat *et al.*, 2009	Reverse	ATTGTAATAGAAGCAGCCCAG
Aquaporin PIP2;1 (VvPIP 2;1)	VIT_13s0019g04280	Forward	CCATTTTGATACCTTCTTCC
	Choat *et al.*, 2009	Reverse	TATCTACAATTTCATGCCCTC
Aquaporin PIP2;4N (VvPIP 2;4N)	VIT_06s0004g02850	Forward	CTAGGATCTTTCAGGAGCAA
	Perrone *et al.*, 2012a	Reverse	TACTCCTCCACCATTGATGT

Fig. S1 RT-PCR analysis on microdissected samples using primers for *VvEF1-α* gene, as housekeeping gene. Any amplified product in RT- reactions excludes DNA contaminations. IRR, irrigated control; WS, water stress; RWS, recovery from water stress; PC, pressure collar stress; RPC, recovery from pressure collar stress.